SPECIFICATION

FLUORESCENT PROTEIN

TECHNICAL FIELD

The present invention relates to a novel fluorescent protein which exists in the form of a monomer. More specifically, the present invention relates to a novel fluorescent protein monomerized by introducing a mutation into a florescent protein derived from Fungia sp., and a use thereof. Further, the present invention relates to a novel chromoprotein and fluorescent protein. More specifically, the present invention relates to a novel chromoprotein and fluorescent protein derived from Montipora. sp, and use thereof.

BACKGROUND ART

Green fluorescent protein (GFP) derived from Aequorea victoria, a jellyfish, has many purposes in biological systems. Recently, various GFP mutants have been produced based on the random mutagenesis and semi-rational mutagenesis, wherein a color is changed, a folding property is improved, luminance is enhanced, or pH sensitivity is modified. Fluorescent proteins such as GFP are fused with other proteins by gene recombinant technique, and monitoring of the expression and transportation of the fusion proteins is carried out.

One of the most commonly used types of GFP mutant is Yellow fluorescent protein (YFP). Among Aequorea-derived GFP mutants, YFP exhibits the fluorescence with the longest wavelength. The values ε and Φ of the majority of YEPs are 60,000 to 100,000 $M^{-1}cm^{-1}$ and 0.6 to 0.8, respectively (Tsien, R. Y. (1998). Ann. Rev. Biochem. 67, 509-544). These values are comparable to those of the general fluorescent group (fluorescein, rhodamine, etc.). Accordingly, improvement of the absolute luminance of YFP is nearly approaching its limit.

In addition, cyan fluorescent protein (CFP) is another example of the GFP mutant. Of this type of protein, ECFP (enhanced cyan fluorescent protein) has been known. Moreover, red fluorescent protein (RFP) has been isolated from sea anemone (Discoma sp.). Of this type of protein, DasRed has been known. Thus, 4 types of fluorescent proteins, that are, green fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, and red fluorescent protein, have successively been developed. The range of the spectrum has significantly been expanded.

Previously, the present inventors had succeeded in amplifying a fluorescent protein gene from among the cDNA library of Fungia sp., using preferred primers designed based on the amino acid sequence of a known fluorescent protein, and then cloning it. Thereafter, the present inventors had determined the fluorescence properties of the obtained fluorescent protein derived from Fungia sp. As a result, the present inventors had found that the above fluorescent protein has desired fluorescence properties (International Publication WO03/54191).

Several GFP homologs derived from Aequorea have a large stokes shift (the difference between an excitation peak value and a fluorescence peak value) (GFPuv and sapphire). These GFP homologs obtain green fluorescence as a result of excitation with UV light at 380 nm. However, the use of such UV light having toxicity is not suitable for observation in organisms. No red fluorescent proteins have a large stokes shift. Under the current circumstances, either excitation or fluorescence must be sacrificed in fluorescence observation.

DISCLOSURE OF THE INVENTION

The molecular weight of the fluorescent protein Kusabira-Orange (KO) isolated from Fungia sp. of Scleractinia, described in International Publication WO03/54191, was measured. As a result, the molecular weight was found to be 70 kDa (the molecular weight calculated from the amino acid sequence thereof was 26 kDa). It is considered

that this fluorescent protein usually forms a dimer. In recent years, the demand for labeling cells or molecules with a fluorescent protein has rapidly grown. When cells are labeled, even if a fluorescent protein forms a multimer, there are no problems because such a fluorescent protein only floats in the cytoplasm. However, when molecules are labeled, such a fluorescent protein that forms a multimer is problematic. For example, when molecules to be labeled form a multimer, there is a possibility that both the target molecule and a fluorescent protein molecule form multimer and that as a result, they form an enormous polymer. In addition, when the formation of a multimer by either one of them is inhibited, such a molecule that cannot form a multimer loses its original properties. Even in a probe of intramolecular FRET (fluorescence resonance energy transfer) wherein multiple fluorescent proteins are used, when fluorescent proteins that form multimer are allowed to express as a single peptide chain, both proteins form multimer, and as a result, the observation of FRET becomes difficult. It is an object of the present invention to solve the aforementioned problems. Specifically, it is an object of the present invention to provide a novel fluorescent protein, which exists in the form of a monomer without forming a multimer.

When compared with a low molecular weight fluorescent substance, a fluorescent protein has broad excitation and fluorescence spectra. Many fluorescent proteins have overlapped portions between such excitation and fluorescence spectra. Thus, it is extremely difficult to excite at an excitation peak value and then to observe at a fluorescence peak value. It is an object of the present invention to provide a fluorescent protein which is able to solve the aforementioned problem. That is to say, it is an object of the present invention to provide a red or orange fluorescent protein, which is characterized in that the difference (stokes shift) between an excitation peak value (wavelength of maximum absorption) and a fluorescence peak value (wavelength of maximum fluorescence) is greatened, so that the maximum fluorescence can be obtained by the maximum excitation.

As a result of intensive studies directed towards achieving the aforementioned objects, the present inventors have succeeded in estimating a multimer interface from the amino acid sequence of the protein KO described in International Publication WO03/54191, substituting amino acids on such a multimer interface with other amino acids, and further in monomerizing KO, so that it can maintain fluorescence properties. Moreover, the present inventors have examined the fluorescence properties of the obtained monomer fluorescent protein. As a result, they have found that it has desired fluorescence properties. The present invention has been completed based on these findings.

Furthermore, as a result of intensive studies directed towards achieving the aforementioned objects, the present inventors have attempted to isolate a gene encoding a novel chromoprotein using Montipora sp. as a material, so as to obtain a chromoprotein COCP. Subsequently, the present inventors have substituted histidine at position 94 of the COCP protein with asparagine, asparagine at position 142 with serine, asparagine at position 157 with aspartic acid, lysine at position 201 with arginine, and phenylalanine at position 206 with serine, so as to produce a fluorescent protein COCP-FL having fluorescence properties. COCP-FL has an excitation peak at 560 nm, and because of this excitation, the peak of the fluorescence spectrum was obtained at 600 nm. Further, the present inventors have substituted serine at position 61 of the aforementioned COCP-FL with phenylalanine, isoleucine at position 92 with threonine, valine at position 123 with threonine, phenylalanine at position 158 with tyrosine, valine at position 191 with isoleucine, and serine at position 213 with alanine, so as to produce a protein keima 616, which has fluorescence properties that are different from those of COCP-FL. Such keima 616 has an excitation peak at 440 nm, and because of this excitation, the peak of the fluorescence spectrum was obtained at 616 nm. Its stokes shift was 176 nm, which was an extremely large value. Still further, the present inventors have substituted phenylalanine at position 61 of keima 616 with methionine, and glutamine at position 62

with cysteine, so as to produce a fluorescent protein keima 570. Such keima 570 has an excitation peak at 440 nm as with keima 616, and because of this excitation, it has a fluorescence peak at 570 nm. Its stokes shift was 130 nm, which was a large value. The present invention has been completed based on these findings.

Thus, the present invention provides a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

Another aspect of the present invention provides a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9, and which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9, respectively.

Further another aspect of the present invention provides a a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, and which has

fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, respectively.

Further another aspect of the present invention provides DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

Further another aspect of the present invention provides DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9, and which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9, respectively.

Further another aspect of the present invention provides DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, and which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, respectively.

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 2; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 2 and that exists in the form of a monomer.

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 4, 6, 8 or 10; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 4, 6, 8 or 10, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 4, 6, 8 or 10, respectively.

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30, respectively.

Further, the present invention provides a chromoprotein described in the

following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 37; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 37, and which has light-absorbing properties.

Another aspect of the present invention provides a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 39; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 39, and which has fluorescence properties.

Further another aspect of the present invention provides a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 41, 43, 45, or 47; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 41, 43, 45, or 47, which has fluorescence properties, and which has a stokes shift of 100 nm or greater.

Further another aspect of the present invention provides DNA encoding a chromoprotein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 37; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 37, and which has light-absorbing properties.

Further another aspect of the present invention provides DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 39; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 39, and which has fluorescence properties.

Further another aspect of the present invention provides DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 41, 43, 45, or 47; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 41, 43, 45, or 47, which has fluorescence properties, and which has a stokes shift of 100 nm or greater.

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 38; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 38, and which has a nucleotide sequence encoding a protein that has light-absorbing properties.

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 40; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 40, and which has a nucleotide sequence encoding a protein that has fluorescence properties.

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 42, 44, 46 or 48; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 42, 44, 46 or 48, and which has a nucleotide sequence encoding a protein that has fluorescence properties and has a stokes shift of 100 nm or greater.

Further another aspect of the present invention provides a recombinant vector having the DNA according to the present invention as mentioned above.

Further another aspect of the present invention provides a transformant having the DNA or the recombinant vector according to the present invention as mentioned above.

Further another aspect of the present invention provides a fusion protein, which consists of the protein according to the present invention as mentioned above and another protein. Preferably, said another protein is a protein that localizes in a cell. More preferably, said another protein is a protein specific to a cell organella. Preferably, said another protein is a fluorescent protein. In this case, preferably, the fusion protein can generate intramolecular FRET.

Further another aspect of the present invention provides a method for analyzing the localization or dynamics of a protein in a cell, which is characterized in that the fusion protein according to the present invention as mentioned above is allowed to express in the cell.

Further another aspect of the present invention provides a reagent kit, which comprises: the fluorescent protein, the DNA, the recombinant vector, the transformant or the fusion protein according to the present invention as mentioned above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the absorption spectrum of mKO.

Figure 2 shows the excitation spectrum (dotted line) and fluorescence spectrum (solid line) of mKO.

Figure 3 shows the results of molecular weight measurement by ultracentrifugation. From the measurement results, the molecular weight was found to be 28 kDa.

Figure 4 shows the results obtained by labeling the mitochondria of HeLa cells with KO (dimer). The mitochondria were converted to granules, which differ from the form of normal mitochondria.

Figure 5 shows the results obtained by labeling the mitochondria of HeLa cells with mKO (monomer). The mitochondria had a normal corded form.

Figure 6 shows the absorption spectrum of a UV-excited green fluorescent mutant mKVU-1.

Figure 7 shows the excitation spectrum and fluorescence spectrum of a UV-excited green fluorescent mutant mKVU-1.

Figure 8 shows the absorption spectrum of a blue fluorescent mutant mKUV-2.

Figure 9 shows the excitation spectrum and fluorescence spectrum of a blue fluorescent mutant mKUV-2.

Figure 10 shows the absorption spectrum of a green fluorescent mutant mKO-FM32.

Figure 11 shows the excitation spectrum and fluorescence spectrum of a green fluorescent mutant mKO-FM32.

Figure 12 shows the absorption spectrum of a red fluorescent mutant mKO-F90.

Figure 13 shows the excitation spectrum and fluorescence spectrum of a red fluorescent mutant mKO-F90.

Figure 14 shows the excitation spectrum at 580nm of mKO time passage mutant

Figure 15 shows the excitation spectrum at 580nm of mKO time passage mutant

Figure 16 shows the fluorescence spectrum of mKO time passage mutant

Figure 17 shows the fluorescence spectrum of mKO time passage mutant

Figure 18 shows the fluorescence spectrum of mKO time passage mutant

Figure 19 shows the fluorescence spectrum of mKO time passage mutant

Figure 20 shows the fluorescence spectrum of mKO time passage mutant

Figure 21 shows the fluorescence spectrum of mKO time passage mutant

Figure 22 shows the fluorescence spectrum of mKO time passage mutant

Figure 23 shows the excitation spectrum at 580 nm obtained until 25 hours after the synthesis of mKO.

Figure 24 shows the excitation spectrum at 580 nm obtained until 25 hours after the synthesis of mKO time passage mutant.

Figure 25 shows the excitation spectrum at 580 nm obtained until 25 hours after the synthesis of mKO time passage mutant.

Figure 26 shows the excitation spectrum at 580 nm obtained until 25 hours after the synthesis of mKO time passage mutant.

Figure 27 shows the excitation spectrum at 580 nm obtained until 25 hours after the synthesis of mKO time passage mutant.

Figure 28 shows the excitation spectrum at 580 nm obtained until 25 hours after the synthesis of mKO time passage mutant.

Figure 29 is a plot showing the ratio between 500 nm as an excitation peak of green fluorescence and 548 nm as an excitation peak of orange fluorescence in an mKO time passage mutant.

Figure 30 shows the results obtained by introducing into HeLa-S3 cells, a fusion protein gene obtained by ligating Tau to the N-terminus of mKO-FM14, followed by imaging.

Figure 31 shows the results obtained by applying strong green light to the mKO protein and measuring the absorption spectrum before and after application of the light.

Figure 32 shows the results obtained by applying strong green light to the mKO-FM14 protein and measuring the absorption spectrum before and after application of the light.

Figure 33 shows the results obtained by detecting under a fluorescence microscope, a fusion protein expressing in cells approximately 12 hours to 2 days after introduction of a BDNF-mKO-FM14 expression gene vector into the cells, then applying strong green light thereto, and then fading orange fluorescence only in a specific region.

Figure 34 shows the results obtained by detecting under a fluorescence microscope, a fusion protein expressing in cells approximately 12 hours to 2 days after introduction of a BDNF-mKO-FM14 expression gene vector into the cells, and then tracing the molecule (BDNF-mKO-FM14) based on change in color.

Figure 35 shows a schematic diagram of a fluorescent protein for carrying out intramolecular FRET.

Figure 36 shows the fluorescence spectrum and absorption spectrum of each of a monomer fluorescent protein mKO and a dimer fluorescent protein MiCy.

Figure 37 shows the measurement results obtained by exciting at 440 nm, the fluorescence spectrum of a reaction solution before and after the reaction of MiCy-linker-mKO with Caspase-3.

Figure 38 shows the results obtained by measuring *in vivo* the activity of Caspase-3 using MiCy-linker-mKO.

Figure 39 shows the absorption spectrum of COCP.

Figure 40 shows the results obtained by measuring the pH sensitivity of COCP.

Figure 41 shows the excitation spectrum and fluorescence spectrum of keima 616.

Figure 42 shows the excitation spectrum and fluorescence spectrum of keima 570.

Figure 43 shows the results obtained by measuring the pH sensitivity of keima 616.

Figure 44 shows the results obtained by measuring the pH sensitivity of keima 570.

Figure 45 shows the results obtained by measuring the molecular weight of cmkeima 620 by ultracentrifugation.

Figure 46 shows the absorption spectrum of cmkeima 620.

Figure 47 shows the absorption spectrum of mkeima 620.

Figure 48 shows the excitation spectrum and fluorescence spectrum of each of keima 616 and ECFP.

Figure 49 shows a protein motif used in the measurement of the activity of Caspase-3.

Figure 50 shows a cross correlation with *in vitro* protease activity. Three types of tandem fluorescent protein samples obtained by insertion of the DEVD sequence into a linker portion were prepared: ECFP-keima 616, keima 616-ECFP, and EGFP-mRFP1 (x 2). The upper case indicates autocorrelation and cross correlation functions obtained before addition of Caspase-3. The middle case indicates a cross correlation function obtained after addition of Caspase-3. The lower case indicates fluorescence intensity obtained after addition of Caspase-3.

Figure 51 shows a relative amplitude in each fusion protein motif.

Figure 52 shows detection of the cleavage of a peptide chain with Caspase-3 (SDS-PAGE).

Figure 53 shows a fusion protein motif used in detection of an interaction between proteins.

Figure 54 shows the fluorescence cross correlation function of ECFP-CaM and M13-keima 616 when $CaCl_2$ is (+).

Figure 55 shows the fluorescence cross correlation function of ECFP-CaM and M13-keima 616 when $CaCl_2$ is (-).

BEST MODE FOR CARRYING OUT THE INVENTION

The embodiments of the present invention will be described in detail below.

(1) Fluorescent proteins of the present invention

(i) The first type of fluorescent protein of the present invention

The first type of fluorescent protein of the present invention is a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

The fluorescent protein of the present invention is characterized in that it has the following properties:

(1) the excitation maximum wavelength is 548 nm, and the fluorescence maximum wavelength is 559 nm;

(2) the molar absorption coefficient at 548 nm is 51,600;

(3) the quantum yield is 0.6; and

(4) the pH sensitivity of fluorescent property is pKa =5.0.

Fungia sp. is a certain type of coral. Fungia sp. is characterized in that it lives mainly in the western area of the Atlantic Ocean, in that the contour of a colony thereof is polygonal, in that it has long tentacles, and in that the body as a whole presents bright orange color.

In the examples given below of the present specification, Fungia sp. was used as a starting material, and the fluorescent protein of the present invention having the aforementioned properties was obtained. However, there are cases where the fluorescent protein of the present invention can also be obtained from coral emitting fluorescence other than Fungia sp. The thus obtained fluorescent protein is also included in the scope of the present invention.

The scope of "one or several" in the phrase "an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids" used herein is not particularly limited. For example, it means 1 to 20, preferably 1 to 10, more preferably 1 to 7, further preferably 1 to 5, and particularly preferably 1 to 3.

The term "equivalent fluorescence properties" is used in the present specification to mean that a fluorescent protein has equivalent fluorescence intensity, equivalent excitation wavelength, equivalent fluorescence wavelength, equivalent pH sensitivity, and the like.

The method of obtaining the fluorescent protein of the present invention is not particularly limited. The proteins may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed by using information regarding the amino acid sequence and the nucleotide sequence shown in SEQ ID NOS 1 to 30 of the sequence listing of the present specification. Using these primers, PCR is carried out by using cDNA clone of the fluorescent protein described in International Publication WO03/54191 as a template, so that DNA encoding the fluorescent protein of the present invention can be obtained. Where a partial fragment of DNA encoding the fluorescent protein of the present invention is obtained by the above-described PCR, the produced DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fluorescent protein can be obtained. The fluorescent protein of the present invention can be produced by introducing this DNA into an appropriate expression system. Expression in an expression system will be described later in the present specification.

In addition, the present invention also provides a mutant protein of the aforementioned protein (mKO) of the present invention. Specifically, there is provided a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9, and which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 3, 5, 7 or 9, respectively.

As further another example, there is provided a a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, and which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, respectively.

(ii) The second type of protein of the present invention

The second type of proteins of the present invention are: a protein having the amino acid sequence shown in SEQ ID NO: 37, 39, 41, 43, 45, or 47; and a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 37, 39, 41, 43, 45, or 47, and which has absorption properties or fluorescence properties. The stokes shifts (the difference between the wavelength of maximum absorption and the wavelength of maximum fluorescence) of the proteins having the amino acid sequence shown in SEQ ID NO: 41, 43, 45, or 47, are 176 nm, 130 nm, 180 nm, and 180 nm, respectively. The stokes shifts of the proteins, which have an amino acid sequence comprising a deletion, substitution, and/or addition of one or several

amino acids with respect to the amino acid sequence shown in SEQ ID NO: 41, 43, 45, or 47, and which have fluorescence properties, are 100 nm or greater, and more preferably 120 nm or greater.

The proteins of the present invention are characterized in that they have the following properties:

(1) COCP (the amino acid sequence thereof is shown in SEQ ID NO: 37, and the nucleotide sequence thereof is shown in SEQ ID NO: 38)

Wavelength of maximum excitation (wavelength of maximum absorption): 576 nm

Molar absorption coefficient at 576 nm: 64,000

pH sensitivity: absent

(2) COCP-FL (the amino acid sequence thereof is shown in SEQ ID NO: 39, and the nucleotide sequence thereof is shown in SEQ ID NO: 40)

Wavelength of maximum excitation (wavelength of maximum absorption): 560 nm

Wavelength of maximum fluorescence: 600 nm

(3) keima 616 (the amino acid sequence thereof is shown in SEQ ID NO: 41, and the nucleotide sequence thereof is shown in SEQ ID NO: 42)

Wavelength of maximum excitation (wavelength of maximum absorption): 440 nm

Wavelength of maximum fluorescence: 616 nm

pH sensitivity: fluorescence intensity is stable between pH 7.5 and 10

(4) keima 570 (the amino acid sequence thereof is shown in SEQ ID NO: 43, and the nucleotide sequence thereof is shown in SEQ ID NO: 44)

Wavelength of maximum excitation (wavelength of maximum absorption): 440 nm

Wavelength of maximum fluorescence: 570 nm

pH sensitivity: fluorescence intensity is stable between pH 7.5 and 10

(5) cmkeima 620 (the amino acid sequence thereof is shown in SEQ ID NO: 45, and the nucleotide sequence thereof is shown in SEQ ID NO: 46)
Wavelength of maximum excitation (wavelength of maximum absorption): 440 nm
Wavelength of maximum fluorescence: 620 nm

(6) mkeima 620 (the amino acid sequence thereof is shown in SEQ ID NO: 47, and the nucleotide sequence thereof is shown in SEQ ID NO: 48)
Wavelength of maximum excitation (wavelength of maximum absorption): 440 nm
Wavelength of maximum fluorescence: 620 nm

In the examples of the present specification, DNA encoding the protein of the present invention was cloned from Montipora sp. used as a starting material. Montipora sp. is a certain type of coral, which belongs to Acropora, Scleractinia, Hexacorallia, Anthozoa, Cnidaria. It often forms an aggregated or coated colony. It is to be noted that the protein of the present invention can also be obtained from coral emitting fluorescence other than Montipora sp. in some cases, and that such a protein is also included in the scope of the present invention.

The scope of "one or several" in the phrase "an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids" used herein is not particularly limited. For example, it means 1 to 20, preferably 1 to 10, more preferably 1 to 7, further preferably 1 to 5, and particularly preferably 1 to 3.

The term "protein having light-absorbing properties" is used to mean in the present specification to mean a protein having properties capable of absorbing light with a certain wavelength. The light-absorbing properties of a "protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 37, and

which has light-absorbing properties" may be either substantially identical to, or different from those of the protein having the amino acid sequence shown in SEQ ID NO: 37. Such light-absorbing properties can be evaluated based on absorption intensity, excitation wavelength (absorption wavelength), pH sensitivity, etc., for example. Among the proteins of the present invention, chromoproteins, which have light-absorbing properties and do not emit fluorescence, can be used, for example, as (1) an FRET acceptor molecule (energy receptor), or can be used in (2) the development of a system for converting irradiated light energy to energy other than light, or in (3) introduction of a mutation into the amino acid sequence of a protein to modify it, so that it can emit fluorescence.

The term "protein having fluorescence properties" is used in the present specification to mean a protein having properties capable of emitting fluorescence as a result of excitation with light having a certain wavelength. The fluorescence properties of the "proteins, which have an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 39, 41, 43, 45, or 47, and which have fluorescence properties" may be either identical to or different from the fluorescence properties of the proteins having the amino acid sequence shown in SEQ ID NO: 39, 41, 43, 45, or 47. Such fluorescence properties can be evaluated based on fluorescence intensity, excitation wavelength, fluorescence wavelength, pH sensitivity, etc., for example.

The method of obtaining the fluorescent protein and the chromoproteins of the present invention is not particularly limited. The proteins may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed by using information regarding the amino acid sequence shown in SEQ ID NO: 37, 39, 41, 43, 45 or 47 of the sequence

listing of the present specification and the nucleotide sequence shown in SEQ ID NO: 38, 40, 42, 44, 46 or 48 thereof. Using these primers, PCR is carried out by using cDNA library derived from Montipora sp. as a template, so that DNA encoding the protein of the present invention can be obtained. Where a partial fragment of DNA encoding the protein of the present invention is obtained by the above-described PCR, the produced DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired protein can be obtained. The protein of the present invention can be produced by introducing this DNA into an appropriate expression system. Expression in an expression system will be described later in the present specification.

(2) DNA of the present invention

The present invention provides genes encoding the first type of fluorescent protein of the present invention.

A specific example of DNA encoding the first type of fluorescent protein of the present invention is DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

A further example of DNA encoding the fluorescent protein of the present invention is DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 2; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in

SEQ ID NO: 2, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 2 and that exists in the form of a monomer.

Further, the DNA encoding the mutant protein of the protein (mKO) of the present invention as described in (1) above, is also included in the scope of the present invention.

Further, the present invention provides genes encoding the second type of protein of the present invention.

A specific example of DNA encoding the protein of the present invention is

(a) a protein having the amino acid sequence shown in SEQ ID NO: 37, 39, 41, 43, 45, or 47; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 37, 39, 41, 43, 45, or 47, and which has light-absorbing properties or fluorescence properties.

Further specific example of DNA encoding the chromoprotein or fruolescent protein of the present invention is DNA described in the following (a) or (b)

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 38, 40, 42, 44, 46 or 48; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 38, 40, 42, 44, 46 or 48, and which has a nucleotide sequence encoding a protein that has light-absorbing properties or fluorescence properties.

In the term "a nucleotide sequence comprising a deletion, substitution and/or addition of one or several nucleotides" used in the present specification, the range of "one or several" is not particularly limited, but is, for example, from 1 to 50, preferably 1 to 30, more preferably 1 to 20, still more preferably 1 to 10, and particularly preferably 1

to 5.

The DNA of the present invention can be synthesized by, for example, the phosphoamidite method, or it can also be produced by polymerase chain reaction (PCR) using specific primers. The DNA of the present invention or its fragment is produced by the method described above in the specification.

A method of introducing a desired mutation into a certain nucleic acid sequence is known to a person skilled in the art. For example, known techniques such as a site-directed mutagenesis, PCR using degenerated oligonucleotides, or the exposure of cells containing nucleic acid to mutagens or radioactive rays, are appropriately used, so as to construct DNA having a mutation. Such known techniques are described in, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989; and Current Protocols in Molecular Biology, Supplements 1 to 38, John Wiley & Sons (1987-1997).

(3) Recombinant vector of the present invention

The DNA of the present invention can be inserted into a suitable vector and used. The type of a vector used in the present invention is not particularly limited. For example, it may be either a vector that can autonomously replicate (e.g., a plasmid, etc.), or vector that is incorporated into the genomes of host cells when it is introduced into the host cells and is then replicated together with the chromosome into which it is incorporated.

The vector used in the present invention is preferably an expression vector. In an expression vector, elements necessary for transcription (e.g., a promoter, etc.) are functionally ligated to the DNA of the present invention. The promoter is a DNA sequence which shows a transcriptional activity in host cells, and it is appropriately selected depending on the type of host cells.

Examples of a promoter which can operate in bacterial cells may include a

Bacillus stearothermophilus maltogenic amylase gene promoter, a *Bacillus licheniformis* alpha-amylase gene promoter, a *Bacillus amyloliquefaciens* BAN amylase gene promoter, a *Bacillus subtilis* alkaline protease gene promoter, a *Bacillus pumilus* xylosidase gene promoter, P_R and P_L promoters of phage rhamda, and lac, trp and tac promoters of *Escherichia coli*.

Examples of a promoter which can operate in mammalian cells may include an SV40 promoter, an MT-1 (metallothionein gene) promoter, and an adenovirus-2 major late promoter. Examples of a promoter which can operate in insect cells may include a polyhedrin promoter, a P10 promoter, an *Autographa californica* polyhedrosis basic protein promoter, a baculovirus immediate-early gene 1 promoter, and a baculovirus 39K delayed-early gene promoter. Examples of a promoter which can be operate in yeast host cells may include promoters derived from yeast glycolytic genes, an alcohol dehydrogenase gene promoter, a TPI1 promoter, and an ADH2-4c promoter.

Examples of a promoter which can operate in filamentous cells may include an ADH3 promoter and a tpiA promoter.

In addition, an appropriate terminator such as a human growth hormone terminator, or a TPI1 terminator or ADH3 terminator for fungal cells, may be functionally bound to the DNA of the present invention, as necessary. The recombinant vector of the present invention may further have elements such as a polyadenylation signal (e.g., one derived from SV40 or the adenovirus 5E1b region), a transcription enhancer sequence (e.g., an SV40 enhancer), or a translation enhancer sequence (e.g., one encoding the adenovirus VA RNA).

The recombinant vector of the present invention may further comprise a DNA sequence which enables the replication of the recombinant vector in host cells. SV40 replication origin is an example of such a sequence (when the host cells are mammalian cells).

The recombinant vector of the present invention may further comprise a

selective marker. Examples of such a selective marker may include genes, complements of which are absent from host cells, such as a dihydrofolate reductase (DHFR) gene or a *Shizosaccharomyces pombe* TPI gene, and drug resistant genes such as ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin or hygromycin-resistant genes.

A method for ligating the DNA of the present invention, a promoter and, as desired, a terminator and/or a secretory signal sequence to one another and inserting these items into a suitable vector is known to a person skilled in the art.

(4) Transformant of the present invention

A transformant can be produced by introducing the DNA or recombinant vector of the present invention into a suitable host.

Any cell can be used as a host cell into which the DNA or recombinant vector of the present invention is introduced, as long as the DNA construct of the present invention can be expressed therein. Examples of such a cell may include bacteria, yeasts, fungal cells, and higher eukaryotic cells.

Examples of bacteria may include Gram-positive bacteria such as Bacillus or Streptomyces, and Gram-negative bacteria such as *Escherichia coli*. These bacteria may be transformed by the protoplast method or other known methods, using competent cells.

Examples of mammalian cells may include HEK 293 cells, HeLa cells, COS cells, BHK cells, CHL cells, and CHO cells. A method of transforming mammalian cells and expressing the introduced DNA sequence in the cells is also known. Examples of such a method may include the electroporation, the calcium phosphate method, and the lipofection method.

Examples of yeast cells may include those belonging to Saccharomyces or Shizosaccharomyces. Examples of such cells may include *Saccharomyces cerevisiae*

and *Saccharomyces kluyveri*. Examples of a method of introducing a recombinant vector into yeast host cells may include the electroporation, the spheroplast method, and the lithium acetate method.

Examples of other fungal cells may include those belonging to *Filamentous fungi* such as Aspergillus, Neurospora, Fusarium or Trichoderma. Where *Filamentous fungi* are used as host cells, transformation can be carried out by incorporating DNA constructs into host chromosomes, so as to obtain recombinant host cells. Incorporation of DNA constructs into the host chromosomes is carried out by known methods, and such known methods may include homologous recombination and heterologous recombination.

Where insect cells are used as host cells, both a vector into which a recombinant gene is introduced and a baculovirus are co-introduced into insect cells, and a recombinant virus is obtained in the culture supernatant of the insect cells. Thereafter, insect cells are infected with the recombinant virus, so as to allow the cells to express proteins (described in, for example, Baculovirus Expression Vectors, A Laboratory Manual; and Current Protocols in Molecular Biology, Bio/Technology, 6, 47 (1988)).

The *Autographa californica* nuclear polyhedrosis virus, which is a virus infecting to insects belonging to *Barathra brassicae*, can be used as baculovirus.

Examples of insect cells used herein may include Sf9 and Sf21, which are *Spodoptera frugiperda* ovarian cells [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman & Company, New York, (1992)], and HiFive (manufactured by Invitrogen), which are *Trichoplusia ni* ovarian cells.

Examples of the method of co-introducing both a vector into which a recombinant gene has been introduced and the above baculovirus into insect cells to prepare a recombinant virus may include the calcium phosphate method and the lipofection method.

The above transformant is cultured in an appropriate nutritive medium under

conditions enabling the introduced DNA construct to be expressed. In order to isolate and purify the protein of the present invention from the culture product of the transformant, common methods of isolating and purifying proteins may be used.

For example, where the protein of the present invention is expressed in a state dissolved in cells, after completion of the culture, cells are recovered by centrifugal separation, and the recovered cells are suspended in a water type buffer. Thereafter, the cells are disintegrated using an ultrasonic disintegrator or the like, so as to obtain a cell-free extract. A supernatant is obtained by centrifuging the cell-free extract, and then, a purified sample can be obtained from the supernatant by applying, singly or in combination, the following ordinary protein isolation and purification methods: the solvent extraction, the salting-out method using ammonium sulfate or the like, the desalting method, the precipitation method using an organic solvent, the anion exchange chromatography using resins such as diethylaminoethyl (DEAE) sepharose, the cation exchange chromatography using resins such as S-Sepharose FF (manufactured by Pharmacia), the hydrophobic chromatography using resins such as butyl sepharose or phenyl sepharose, the gel filtration method using a molecular sieve, the affinity chromatography, the chromatofocusing method, and the electrophoresis such as isoelectric focusing.

(5) Use of the fluorescent protein of the present invention and a fusion fluorescent protein comprising the same

The fluorescent protein of the present invention can be fused with another protein, so as to construct a fusion fluorescent protein.

A method of obtaining the fusion fluorescent protein of the present invention is not particularly limited. It may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA

encoding the protein. Appropriate primers are designed using the information regarding the amino acid sequence and the nucleotide sequence shown in SEQ ID NOS: 1 to 30 of the sequence listing of the present specification. Using these primers, PCR is carried out using a DNA fragment containing the gene of the fluorescent protein of the present invention as a template, so as to produce DNA fragments necessary for construction of the DNA encoding the fluorescent protein of the present invention. Moreover, DNA fragment encoding a protein to be fused is also obtained in the same above manner.

Subsequently, the thus obtained DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fusion fluorescent protein can be obtained. This DNA is then introduced into an appropriate expression system, so that the fusion fluorescent protein of the present invention can be produced.

The fluorescent protein of the present invention has an extremely high utility value as a marker. This is to say, the fluorescent protein of the present invention is purified as a fusion protein with an amino acid sequence to be tested, and the fusion protein is introduced into cells by methods such as the microinjection. By observing the distribution of the fusion protein over time, targeting activity of the amino acid sequence to be tested can be detected in the cells.

The type of another protein (an amino acid sequence to be tested) with which the fluorescent protein of the present invention is fused is not particularly limited. Preferred examples may include proteins localizing in cells, proteins specific for intracellular organelles, and targeting signals (e.g., a nuclear transport signal, a mitochondrial presequence, etc.). In addition, the fluorescent protein of the present invention can be expressed in cells and used, as well as being introduced into cells by the microinjection or the like. In this case, a vector into which the DNA encoding the fluorescent protein of the present invention is inserted in such a way that it can be expressed, is introduced into host cells.

Moreover, the fluorescent protein of the present invention can also be used as a

reporter protein to determine promoter activity. This is to say, a vector is constructed such that DNA encoding the fluorescent protein of the present invention is located downstream of a promoter to be tested, and the vector is then introduced into host cells. By detecting the fluorescence of the fluorescent protein of the present invention which is emitted from the cells, the activity of the promoter to be tested can be determined. The type of a promoter to be tested is not particularly limited, as long as it operates in host cells.

A vector used to detect the targeting activity of the above amino acid sequence to be tested or to determine promoter activity is not particularly limited. Examples of a vector preferably used for animal cells may include pNEO (P. Southern, and P. Berg (1982) J. Mol. Appl. Genet. 1: 327), pCAGGS (H. Niwa, K. Yamamura, and J. Miyazaki, Gene 108, 193-200 (1991)), pRc/CMV (manufactured by Invitrogen), and pCDM8 (manufactured by Invitrogen). Examples of a vector preferably used for yeasts may include pRS303, pRS304, pRS305, pRS306, pRS313, pRS314, pRS315, pRS316 (R. S. Sikorski and P. Hieter (1989) Genetics 122: 19-27), pRS423, pRS424, pRS425, pRS426 (T. W. Christianson, R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter (1992) Gene 110: 119-122).

In addition, the type of cells used herein is also not particularly limited. Various types of animal cells such as L cells, BalbC-3T3 cells, NIH3T3 cells, CHO (Chinese hamster ovary) cells, HeLa cells or NRK (normal rat kidney) cells, yeast cells such as *Saccharomyces cerevisiae*, *Escherichia coli* cells, or the like can be used. Vector can be introduced into host cells by common methods such as the calcium phosphate method or the electroporation.

The above obtained fusion fluorescent protein of the present invention wherein the fluorescent protein of the present invention is fused with another protein (referred to as a protein X) is allowed to be expressed in cells. By monitoring a fluorescence emitted, it becomes possible to analyze the localization or dynamics of the protein X in

cells. That is, cells transformed or transfected with DNA encoding the fusion fluorescent protein of the present invention are observed with a fluorescence microscope, so that the localization and dynamics of the protein X in the cells can be visualized and thus analyzed.

For example, by using a protein specific for an intracellular organella as a protein X, the distribution and movement of a nucleus, a mitochondria, an endoplasmic reticulum, a Golgi body, a secretory vesicle, a peroxisome, etc., can be observed.

Moreover, for example, axis cylinders or dendrites of the nerve cells show an extremely complicated change in strikes in an individual who is under development. Accordingly, fluorescent labeling of these sites enables a dynamic analysis.

The fluorescence of the fluorescent protein of the present invention can be detected with a viable cell. Such detection can be carried out using, for example, a fluorescence microscope (Axiophoto Filter Set 09 manufactured by Carl Zeiss) or an image analyzer (Digital Image Analyzer manufactured by ATTO).

The type of a microscope can be appropriately selected depending on purposes. Where frequent observation such as pursuit of a change over time is carried out, an ordinary incident-light fluorescence microscope is preferable. Where observation is carried out while resolution is emphasized, for example, in the case of searching localization in cells specifically, a confocal laser scanning microscope is preferable. In terms of maintenance of the physiological state of cells and prevention from contamination, an inverted microscope is preferable as a microscope system. When an erecting microscope with a high-powered lens is used, a water immersion lens can be used.

A filter set can be appropriately selected depending on the fluorescence wavelength of a fluorescent protein. Since the fluorescent protein of the present invention has an excitation maximum wavelength of 548 nm, and a fluorescence maximum wavelength of 559 nm, a filter having an excitation light between

approximately 530 and 550 nm and a fluorescence between approximately 550 and 600 nm is preferably used.

When viable cells are observed over time using a fluorescence microscope, a high sensitive cooled CCD camera is used, since photography is carried out in a short time. In the case of the cooled CCD camera, CCD is cooled to decrease thermal noise, so that a weak fluorescence image can be clearly photographed by exposure in a short time.

FRET (fluorescence resonance energy transfer) has been known as a means for analyzing the interaction between molecules. In FRET, for example, a first molecule labeled with a cyan fluorescent protein (CFP) acting as a first fluorescent protein is allowed to coexist with a second molecule labeled with a yellow fluorescent protein (YFP) acting as a second fluorescent protein, so as to allow the yellow fluorescent protein (YFP) to act as an acceptor molecule and to allow the cyan fluorescent protein (CFP) to act as a donor molecule. Thus, FRET (fluorescence resonance energy transfer) is allowed to take place between both molecules, so as to visualize the interaction between the first and second molecules. Namely, in FRET, different dyes are introduced into two types of molecules. One dye with a higher energy level (a donor molecule) is selectively excited, and the fluorescence of the dye is measured. Long-wavelength fluorescence from the other dye (an acceptor molecule) is also measured. The interaction between the molecules is visualized by using the difference between the amounts of both fluorescences. Only when both dyes are adjacent to each other due to the interaction of the two types of molecules, a decrease in the fluorescence of the donor molecule and an increase in the fluorescence of the acceptor molecule are observed by single wavelength excitation dual wavelength photometry. However, in a case where a chromoprotein is used as an acceptor molecule, a decrease in the fluorescence of the donor molecule occurs only when both dyes are adjacent to each other by the interaction of the two types of molecules. Such a decrease can be observed

by single wavelength excitation single wavelength photometry. Thus, facilitation of measurement apparatuses becomes possible.

The fluorescent protein and chromoprotein and of the present invention is particularly advantageous when it is used as a donor molecule and an acceptor molecule in FRET (fluorescence resonance energy transfer). That is to say, a fused form (a first fused form) of the chromoprotein of the present invention and a test substance is first produced. Then, a fused form (a second fused form) of another test substance interacting with the above test substance and another fluorescent protein is produced. Thereafter, the first fused form is allowed to interact with the second fused form, and the generated fluorescence is analyzed, so that the interaction between the aforementioned two types of test substances can be analyzed. FRET (fluorescence resonance energy transfer) using the chromoprotein of the present invention may be carried out either in a test tube or in a cell.

Further, one or more of either of the fluorescent protein and chromoprotein and of the present invention can be used as a donor protein or an acceptor protein. Thus, a fluorescent indicator having a structure where a donor fruolescent protein and an acceptor fluorescent protein are bound to both ends of a target sequence of an analysed substance respectively, can be prepared. The conformation of the fluorescent indicator is changed based on the presence or absence of binding or action of an analyzed substance to said target sequence, thus the presence or absence of FRET (fluorescence resonance energy transfer) can be generated.

(6) Kit of the present invention

The present invention provides a kit for analyzing the localization of intracellular components and/or analyzing physiologically active substances, which is characterized in that it comprises at least one selected from the fluorescent protein, the fusion fluorescent protein, the DNA, the recombinant vector, or the transformant, which

are described in the present specification. The kit of the present invention can be produced from commonly used materials that are known per se, by using common methods.

Reagents such as the fluorescent protein or the DNA are dissolved in an appropriate solvent, so that the reagents can be prepared in a form suitable for conservation. Water, ethanol, various types of buffer solution, etc. can be used as such a solvent.

The present invention will be further described in the following examples. However, the present invention is not limited by these examples.

EXAMPLES

Example 1: Production of multimer formation-inhibiting mutant by point mutation introduction

A multimer interface was predicted from the amino acid sequence of KO-1, and the amino acids of the multimer interface were substituted with other amino acids. Moreover, KO-1 was monomerized, so that it could maintain fluorescence properties. Point mutation introduction was carried out, using an *Escherichia coli* expression vector (pRSET $_B$) (an expression vector having DNA encoding KO-1 described in International Publication WO03/54191), into which KO-1 had been inserted, and also using point mutation introduction primers. Specifically, multiple mutation introduction primers were simultaneously annealed on one side chain of a template plasmid, followed by elongation with polymerase. DNA fragments elongated with each primers were ligated to one another using DNA ligase in the same reaction solution, so as to obtain a product, whose portions other than the mutation-introduced portion were complementary to the template. Since the termini of the DNA fragments needed phosphate groups when the fragments were ligated to one another with DNA ligase, the 5'-terminal sides of the used primers had been subjected to phosphorylation.

(1) 5'-phosphorylation of primers

100 μM primers	2 μl
10 x T4 polynucleotide kinase buffer	5 μl
100 μM ATP	0.5 μl
Sterilized water	41.5 μl
T4 polynucleotide kinase (10 U/μl)	1 μl

The above mixture was incubated at 37°C for 30 minutes. As primers used herein, the following primers having the nucleotide sequences shown in SEQ ID NOS: 3 to 17 were used.

K11R, F13Y

CCAGAGATGAAGATGAGGTACTACATGGACGGC (SEQ ID NO:59)

V25I

CATGAGTTCACAATTGAAGGTGAAGGC (SEQ ID NO:60)

K32R

GAAGGCACAGGCAGACCTTACGAGGGA (SEQ ID NO:61)

S55A

CCAATGCCTTTCGCGTTTGACTTAGTG (SEQ ID NO:62)

T62V

TTAGTGTCACACGTGTTCTGTTACGGC (SEQ ID NO:63)

Q96E

GAAAGGTCGTTGGAGTTCGAAGATGGT (SEQ ID NO:64)

F102S, A104S

GAAGATGGTGGGTCCGCTTCAGTCAGTGCG (SEQ ID NO:65)

C115T, E117Y

AGCCTTAGAGGAAACACCTTCTACCACAAATCCA (SEQ ID NO:66)

V123T

CAAATCCAAATTTACTGGGGTTAACTTTCCTG (SEQ ID NO:67)

V133I

GCCGATGGTCCTATCATGCAAAACCAAAGT (SEQ ID NO:68)

S139V

GCCGATGGTCCTATCATGCAAAACCAAAGTGTTGATTGGGAGCCA (SEQ ID NO:69)

T150A, C151S

GAGAAAATTACTGCCAGCGACGGAGTTCTGAAG (SEQ ID NO:70)

F162Y, A166E

GATGTTACGATGTACCTAAAACTTGAAGGAGGCGGCAATCAC (SEQ ID NO:71)

Q190G, F193Y, G195S

CTTAAAATGCCAGGAAGCCATTACATCAGCCATCGCCTCGTCAGG (SEQ ID NO:72)

C217S

GATGCAGTAGCTCATTCCCTCGAGCACCACCACC (SEQ ID NO:73)

(2) Point mutation introduction PCR

5'-phosphorylated primers	4 μl
Template (KO-pRSET $_B$)	100 ng
10 x polymerase buffer	2.5 μl
10 x DNA ligase buffer	2.5 μl
2.5 mM dNTPs	1 μl
polymerase (pfu) 2.5U/μl	1 μl
Taq DNA ligase 40U/μl	0.5 μl

The final volume of the mixture was set at 50 μl by addition of sterilized water.

Program:

GeneAmp PCR system 9700 was used as a thermal cycler.

(1) 65°C 5 min

(2) 95°C 2 min

(3) 95°C 20 sec

(4) 52°C 20 sec

(5) 65°C 8 min

The operation described in (3) to (5) above was repeated for 25 cycles.

(6) 75°C 7 min

(7) 4°C hold

(3) Dpn1 treatment

1 μl of Dpn1 was added to the sample obtained after PCR, and the obtained mixture was then incubated at 37°C for 1 hour, so as to cleave a template plasmid.

(4) Transformation of *Escherichia coli*

Escherichia coli JM109 was transformed with the sample treated with Dpn1, so that KO-1 after introduction of the mutation was allowed to express therein.

(5) Amino acid sequence of monomerized Kusabira-Orange (mKO)

The nucleotide sequence of the KO mutant obtained after introduction of the mutation was analyzed, so as to determine the amino acid sequence thereof. As a result, it was found that lysine (K) at position 11 was substituted with arginine (R), phenylalanine (F) at position 13 was substituted with tyrosine (Y), valine (V) at position 25 was substituted with isoleucine (I), lysine (K) at position 32 was substituted with arginine (R), serine (S) at position 55 was substituted with alanine (A), threonine (T) at position 62 was substituted with valine (V), glutamine (Q) at position 96 was substituted with glutamic acid (E), phenylalanine (F) at position 102 was substituted with serine (S), alanine (A) at position 104 was substituted with serine (S), cysteine (C) at position 115 was substituted with threonine (T), glutamic acid (E) at position 117 was substituted with

tyrosine (Y), valine (V) at position 123 was substituted with threonine (T), valine (V) at position 133 was substituted with isoleucine (I), serine (S) at position 139 was substituted with valine (V), threonine (T) at position 150 was substituted with alanine (A), cysteine (C) at position 151 was substituted with serine (S), phenylalanine (F) at position 162 was substituted with tyrosine (Y), alanine (A) at position 166 was substituted with glutamic acid (E), glutamine (Q) at position 190 was substituted with glycine (G), phenylalanine (F) at position 193 was substituted with tyrosine (Y), glycine (G) at position 195 was substituted with serine (S), and cysteine (C) at position 217 was substituted with serine (S). Moreover, in order to add the Kozak sequence, valine (V) was introduced into the position before serine (S) at position 2. This mutant was named as mKO. The amino acid sequence of mKO is shown in SEQ ID NO: 1 of the sequence listing, and the nucleotide sequence thereof is shown in SEQ ID NO: 2 of the sequence listing.

A protein formed by adding His-Tag to mKO was allowed to express in *Escherichia coli* according to common methods, and it was then purified with Ni-Agarose.

Example 2: Analysis of fluorescence properties

The fluorescence and absorption spectra of the mKO protein purified in Example 1 were measured as follows. The quantum yield and molar absorption coefficient thereof were calculated.

An absorption spectrum was measured using a 20 μM fluorescent protein and a 50 mM HEPES solution (pH 7.5). A molar absorption coefficient was calculated from the peak value of this spectrum. In the case of mKO, the fluorescent protein was diluted with the aforementioned buffer solution such that the absorption peak was found at 548 nm and such that absorption at 500 nm became 0.0025. Thereafter, the fluorescence spectrum obtained by excitation at 500 nm, and the excitation spectrum

obtained by fluorescence at 590 nm, were measured. The fluorescence spectrum of DsRed (CLONTECH) was also measured under conditions wherein the absorption at 500 nm became 0.0025. The quantum yield of DsRed was set at 0.29, and the quantum yield of mKO was obtained.

The results are shown in Table 1, and Figures 1 and 2. Table 1 also shows the data of the KO protein (dimer protein) described in International Publication WO03/54191.

Table 1

	Maximum excitation	Maximum fluorescence	Molar absorption coefficient	Quantum yield	Number of amino acids	multimer formation	pH sensitivity
KO	548 nm	561 nm	109750	0.45	217	Dimer	pKa<5.0
mKO	548 nm	559 nm	51600	0.6	218	Monomer	PKa=5.0

Example 3: Measurement of molecular weight by ultracentrifugal analysis

An mKO protein solution with the composition consisting of 150 mM KCl and 50 mM HEPES-KOH (pH 7.4) was prepared. The molecular weight of mKO was determined by ultracentrifugal analysis. The above solution was centrifuged with an ultracentrifuge XL-1 (Beckman Coulter) at 25,000 rpm for 22 hours, so as to measure absorption at 540 nm around the maximum absorption (548 nm) of mKO. From the measurement results, the molecular weight of mKO was calculated to be 28 kDa (Figure 3). This value was almost the same as 26 kDa predicted from the amino acid sequence, and thus it was confirmed that mKO exists in the form of a monomer.

Example 4: Targeting to mitochondria

12 amino acids (MLSLRQSIRFFK) at the N-terminus of cytochrome oxidase

subunit 4 derived from yeast were added to each of the N-termini of KO and mKO. Thereafter, targeting to the mitochondria of HeLa cells was conducted, so as to label the mitochondria. As a result, it was confirmed that KO (dimer) was not exactly targeted to the mitochondria, and that the mitochondria was stained in a granulated state (Figure 4). On the other hand, mKO (monomer) was exactly targeted to the mitochondria, and narrow filamentous mitochondria were observed. Thus, effectiveness obtained by monomerization was confirmed (Figure 5).

Example 5: Production of mKO mutant having different fluorescence properties

(1) Mutation introduction

The amino acids of mKO were substituted with other amino acids, so as to produce a fluorescent protein having fluorescence properties that are different from those of mKO. Point mutation introduction was carried out by performing PCR, using an *Escherichia coli* expression vector ($pRSET_B$), into which mKO had been inserted, and also using point mutation introduction primers. The primers used in PCR had been subjected to 5'-phosphorylation.

(a) 5'-phosphorylation of primers

100 μM primers	2 μl
10 x T4 polynucleotide kinase buffer	5μl
100 μM ATP	0.5μl
Sterilized water	41.5μl
T4 polynucleotide kinase (10 U/μl)	1 μl

The mixture was incubated at 37°C for 30 minutes.

(b) Point mutation introduction PCR

5'-phosphorylated primers	4 μl
Template (mKO-$pRSET_B$)	100 ng

10 x polymerase buffer	2.5 μl
10 x DNA ligase buffer	2.5 μl
2.5 mM dNTPs	1 μl
polymerase (pfu) 2.5 U/μl	1 μl
Taq DNA ligase 40 U/μl	0.5 μl

The final volume of the mixture was set at 50 μl by addition of sterilized water.

Program:

GeneAmp PCR system 9700 was used as a thermal cycler.

(1) 65°C 5 min

(2) 95°C 2 min

(3) 95°C 20 sec

(4) 52°C 20 sec

(5) 65°C 8 min

(6) 75°C 7 min

(7) 4°C hold

The operation described in (3) to (5) above was repeated for 25 cycles.

(c) Dpn1 treatment

1 μl of Dpn1 was added to the sample obtained after PCR, and the obtained mixture was then incubated at 37°C for 1 hour, so as to cleave a template plasmid.

(d) Transformation of *Escherichia coli*

Escherichia coli JM109 (DE3) was transformed with the sample treated with Dpn1, so that mKO after introduction of the mutation was allowed to express therein. Thereafter, mKO was analyzed.

(2) Amino acid-substituted site and fluorescence properties of mKO mutant

A fluorospectrophotometer F-2500 (HITACHI) was used for fluorescence measurement. A spectrophotometer U-3310 (HITACHI) was used for absorption measurement.

(i) UV-excited green fluorescent mutant mKUV-1 (the amino acid sequence thereof is shown in SEQ ID NO: 3, and the nucleotide sequence thereof is shown in SEQ ID NO: 4)

In mKO, proline (P) at position 70 was substituted with cysteine (C), valine (V) at position 160 was substituted with aspartic acid (D), methionine (M) at position 162 was substituted with leucine (L), and phenylalanine (F) at position 176 was substituted with methonine (M), so as to obtain a green fluorescent protein having a fluorescence peak at 505 nm and an excitation peak at 398 nm (Figures 6 and 7). The molar absorption coefficient thereof was 10,000, and the quantum yield of fluorescence was 0.27.

(ii) Blue fluorescent mutant mKUV-2 (the amino acid sequence thereof is shown in SEQ ID NO: 5, and the nucleotide sequence thereof is shown in SEQ ID NO: 6)

In mKO, cysteine (C) at position 65 was substituted with glycine (G), proline (P) at position 70 was substituted with glycine (G), valine (V) at position 160 was substituted with aspartic acid (D), and phenylalanine (F) at position 176 was substituted with methionine (M), so as to obtain a blue fluorescent protein having a fluorescence peak at 469 nm and an excitation peak at 322 nm (Figures 8 and 9). The molar absorption coefficient thereof was 12,500, and the quantum yield of fluorescence was 0.2.

(iii) Green fluorescent mutant mKO-FM32 (the amino acid sequence thereof is shown in SEQ ID NO: 7, and the nucleotide sequence thereof is shown in SEQ ID NO: 8)

In mKO, cysteine (C) at position 65 was substituted with alanine (A), and proline (P) at position 70 was substituted with glycine (G), so as to obtain a green

fluorescent protein having a fluorescence peak at 506 nm and an excitation peak at 493 nm (Figures 10 and 11). The molar absorption coefficient thereof was 27,500, and the quantum yield of fluorescence was 0.44.

(iv) Red fluorescent mutant mKO-F90 (the amino acid sequence thereof is shown in SEQ ID NO: 9, and the nucleotide sequence thereof is shown in SEQ ID NO: 10)

In mKO, methionine (M) at position 41 was substituted with leucine (L), lysine (K) at position 49 was substituted with glutamic acid (E), arginine (R) at position 69 was substituted with lysine (K), serine (S) at position 145 was substituted with tryptophan (W), lysine (K) at position 185 was substituted with glutamic acid (E), lysine (K) at position 188 was substituted with glutamic acid (E), and serine (S) at position 192 was substituted with aspartic acid (D), so as to obtain a red fluorescent protein having a fluorescence peak at 582 nm and an excitation peak at 564 nm (Figures 12 and 13). The molar absorption coefficient thereof was 25,000, and the quantum yield of fluorescence was 0.05.

Example 6: Production of mKO mutant emitting both green and orange fluorescence (time passage measurement probe and trace probe)

The amino acids of mKO were substituted with other amino acids, so as to produce a fluorescent protein having fluorescence properties that are different from those of mKO. Immediately after being translated, mKO emitted green fluorescence, and then emitted orange fluorescence. However, since such a shift from green fluorescence to orange fluorescence has been rapidly completed, it is generally invisible. Thus, a fluorescent protein having different ratios between green fluorescence and orange fluorescence depending on various types of time passage was produced. Using this mutant protein, the time passed after expression of the protein can be measured based on the ratio between the green fluorescence and the orange fluorescence. In addition, since such green fluorescence and orange fluorescence exist independently in the mutant, only

the orange fluorescence could be quenched. That is, if only the orange fluorescence is quenched and an increase in the orange fluorescence is measured, the measurement of time passage can be reset. Moreover, if any given portion of only the orange color is quenched and the time passage is measured based on the ratio between the green fluorescence and the orange fluorescence, it also becomes possible to measure the behavior of the labeled molecules or cells of such a quenched portion. As a result, it was found that a fluorescent protein having various types of ratios between green fluorescence and orange fluorescence as the time passed, can be produced by substituting proline (P) at position 70 with another amino acid.

(1) Mutation introduction

The amino acids of mKO were substituted with other amino acids, so as to produce a fluorescent protein having fluorescence properties that are different from those of mKO. Point mutation was introduced by performing PCR using an *Escherichia coli* expression vector ($pRSET_B$), into which mKO had been inserted, and also using point mutation introduction primers. The primers used in PCR had been subjected to 5'-phosphorylation.

(a) 5'-phosphorylation of primers

100 μM primers	2 μl
10 x T4 polynucleotide kinase buffer	5μl
100 μM ATP	0.5μl
Sterilized water	41.5μl
T4 polynucleotide kinase (10 U/μl)	1 μl

The mixture was incubated at 37°C for 30 minutes.

(b) Point mutation introduction PCR

5'-phosphorylated primers	4 μl
Template (mKO-$pRSET_B$)	100 ng

10 x polymerase buffer	2.5 μl
10 x DNA ligase buffer	2.5 μl
2.5 mM dNTPs	1 μl
polymerase (pfu) 2.5 U/μl	1 μl
Taq DNA ligase 40 U/μl	0.5 μl

The final volume of the mixture was set at 50 μl by addition of sterilized water.

Program:

GeneAmp PCR system 9700 was used as a thermal cycler.

(1) 65°C 5 min

(2) 95°C 2 min

(3) 95°C 20 sec

(4) 52°C 20 sec

(5) 65°C 8 min

(6) 75°C 7 min

(7) 4°C hold

The operation described in (3) to (5) above was repeated for 25 cycles.

(c) Dpn1 treatment

1 μl of Dpn1 was added to the sample obtained after PCR, and the obtained mixture was then incubated at 37°C for 1 hour, so as to cleave a template plasmid.

(d) Transformation of *Escherichia coli*

Escherichia coli JM109 (DE3) was transformed with the sample treated with Dpn1, so that mKO after introduction of the mutation was allowed to express therein. Thereafter, mKO was analyzed.

(2) Analysis of mKO time passage mutant

The nucleotide sequence of the produced mKO mutant was analyzed. As a result, it was found that in the mKO mutant, lysine (K) at position 49 was substituted with glutamic acid (E), proline (P) at position 70 was substituted with glycine (G), lysine (K) at position 185 was substituted with glutamic acid (E), lysine (K) at position 188 was substituted with glutamic acid (E), serine (S) at position 192 was substituted with aspartic acid (D), and serine (S) at position 196 was substituted with glycine (G). This mKO mutant was a fluorescent protein whose ratio between the green fluorescence and the orange fluorescence is changed depending on time passage. By substituting proline (P) at position 70 of this mKO mutant with various types of amino acids, the rate of changing the ratio between the green fluorescence and the orange fluorescence depending on time passage was changed.

A mutant where proline (P) at position 70 was substituted with glycine (G) was referred to as mKO-FM9 (the amino acid sequence thereof is shown in SEQ ID NO: 11, and the nucleotide sequence thereof is shown in SEQ ID NO: 12).

A mutant where proline (P) at position 70 was substituted with alanine (A) was referred to as mKO-FM5 (the amino acid sequence thereof is shown in SEQ ID NO: 13, and the nucleotide sequence thereof is shown in SEQ ID NO: 14).

A mutant where proline (P) at position 70 was substituted with serine (S) was referred to as mKO-FM3 (the amino acid sequence thereof is shown in SEQ ID NO: 15, and the nucleotide sequence thereof is shown in SEQ ID NO: 16).

A mutant where proline (P) at position 70 was substituted with cysteine (C) was referred to as mKO-FM20 (the amino acid sequence thereof is shown in SEQ ID NO: 17, and the nucleotide sequence thereof is shown in SEQ ID NO: 18).

A mutant where proline (P) at position 70 was substituted with threonine (T) was referred to as mKO-FM24 (the amino acid sequence thereof is shown in SEQ ID NO: 19, and the nucleotide sequence thereof is shown in SEQ ID NO: 20).

A mutant where proline (P) at position 70 was substituted with valine (V) was

referred to as mKO-FM14 (the amino acid sequence thereof is shown in SEQ ID NO: 21, and the nucleotide sequence thereof is shown in SEQ ID NO: 22).

A mutant where proline (P) at position 70 was substituted with leucine (L) was referred to as mKO-FM19 (the amino acid sequence thereof is shown in SEQ ID NO: 23, and the nucleotide sequence thereof is shown in SEQ ID NO: 24).

A mutant where proline (P) at position 70 was substituted with tyrosine (Y) was referred to as mKO-FM23 (the amino acid sequence thereof is shown in SEQ ID NO: 25, and the nucleotide sequence thereof is shown in SEQ ID NO: 26).

A mutant where proline (P) at position 70 was substituted with glutamine (Q) was referred to as mKO-FM21 (the amino acid sequence thereof is shown in SEQ ID NO: 27, and the nucleotide sequence thereof is shown in SEQ ID NO: 28).

A mutant where proline (P) at position 70 was substituted with asparagine (N) was referred to as mKO-FM25 (the amino acid sequence thereof is shown in SEQ ID NO: 29, and the nucleotide sequence thereof is shown in SEQ ID NO: 30).

The measurement of each mKO time passage mutant was carried out using a recombinant fluorescent protein that was allowed to express in *Escherichia coli* JM109 (DM3), or using the *in vitro* translation system PURE SYSTEM CLASSIC MINI (Post Genome Institute Co., Ltd.). With regard to measurement in *Escherichia coli*, a culture plate, on which each mutant had been expressed, was incubated at 37°C, and thereafter, sampling was carried out over time, so as to measure an excitation spectrum at 580 nm (Figures 14 and 15). As a result, it was found that the peak at 548 nm that is the excitation peak of orange fluorescence increased over time rather than the peak at approximately 500 nm that is the excitation peak of green fluorescence, and that the increase rate differed depending on the type of each mutant. The peak of the green fluorescence was found to be 509 nm, and the peak of the orange fluorescence was found to be 560 nm (Figures 16, 17, 18, 19, 20, 21, and 22; each of them was excited with the wavelength described in the parenthesis). A fluorospectrophotometer F-2500 (HITACHI)

was used for fluorescence measurement. Since novel proteins are intermittently produced in *Escherichia coli*, the time required for a shift from green to orange is apparently prolonged. Thus, the *in vitro* translation system was used to limit the production time of such proteins, so as to measure a shift from green to orange with more exact time passage. The time required for the synthesis of proteins was set at 1 hour. Immediately after 1 hour had passed, an energy source necessary for the synthesis of proteins, such as ATP, was removed by gel filtration, and the residue was then incubated at 37°C, so as to synthesize proteins. Thereafter, the excitation spectrum at 580 nm was measured until 25 hours after the synthesis (Figures 23, 24, 25, 26, 27, and 28). The ratio between the value at 500 nm of the excitation peak portion of the green fluorescence and the value at 548 nm of the excitation peak portion of the orange fluorescence was plotted. As a result, it was found that as the side chains of amino acids relatively greaten (G→A→S→C→T→V→P), the speed of a shift to the orange fluorescence component tends to become rapid (Figure 29).

A fusion protein gene (whose amino acid sequence is shown in SEQ ID NO: 31, and nucleotide sequence is shown in SEQ ID NO: 32), which had been obtained by genetically ligating Tau (a protein that binds to a tubulin or the like and promotes microtubule polymerization for stabilization) to the N-terminus of mKO-FM14, was subcloned into the BamH1-Xho1 site of the animal cell expression vector pCDNA3. Thereafter, the thus produced vector was introduced into HeLa-S3 cells, using Polyfect (QIAGEN). Twenty-three hours after introduction of the gene, the culture solution was substituted with the HBSS (Hanks' Balanced Salt Solution), followed by imaging. As a result, cells with various types of color tones ranging from green to orange were observed, depending on the difference in time wherein the vector was incorporated into the HeLa-S3 cells. From the ratio between the orange color and the green color, it was confirmed that cells 4 and 5 incorporated the vector therein at an early stage, and that cells 1, 2, and 3 then incorporated the vector therein (Figure 30). IX-70 (OLYMPUS) was used herein as a

microscope. In order to detect the green color component, 470DF35 (OMEGA) was used as an excitation filter, HQ525/50M (CHROMA) was used as a fluorescence filter, and 505DRLP (OMEGA) was used as a dichroic mirror. In addition, in order to detect the orange color component, HQ500/40X (CHROMA) was used as an excitation filter, OG550 (OMEGA) was used as a fluorescence filter, and Q530LP (CHROMA) was used as a dichroic mirror.

(3) Trace of molecules with mKO time passage mutant

A strong green light was applied to a recombinant mKO-FM14 protein, so as to examine whether it was able to discolor only the orange-color fluorescence component of the recombinant mKO-FM14 protein. A filter was directly attached to a 100-W xenon lamp, and a strong green light was then applied to the recombinant mKO-FM14 protein. As a filter, 546DF20 (OMEGA) was used. As a control, such a strong green light was also applied to a recombinant mKO protein. Thereafter, the absorption spectrum was measured before and after application of the light, so as to examine whether the absorption value at 548 nm decreased. A spectrophotometer U-3310 (HITACHI) was used to measure absorption. As a result, the absorption value at 548 nm of the recombinant mKO protein used as a control did not change. In contrast, the absorption value at 548 nm of the recombinant mKO-FM14 protein significantly decreased. However, the absorption peak at 500 nm necessary for emission of a green fluorescence component did not change (Figures 31 and 32). This indicates that only the orange fluorescence component can be eliminated or decreased by application of a strong green light to the mKO-FM14 protein. In addition, if the ratio between the orange fluorescence signal and the green fluorescence signal is calculated based on the quenching or decrease of the orange fluorescence by application of a strong green light to only a localized portion in a space filled with the mKO-FM14 protein or an mKO-FM14 protein-added product, only the portion can be labeled. Thus, a fusion protein gene (the amino acid sequence thereof is shown in SEQ

ID NO: 33, and the nucleotide sequence thereof is shown in SEQ ID NO: 34), which had been obtained by fusion of BDNF (brain-derived neurotrophic factor) to the N-terminus of mKO-FM14, was subcloned into a product obtained by extracting EGFP from pEGFP-N1 (Clontech). The resultant was then allowed to express in rat hippocampus neurons, followed by imaging.

That is, rat hippocampus neurons were prepared. Hippocampal portions (of approximately 10 rats) were excised from fetuses in pregnant rats (17^{th} to 19^{th} day after conception), or from newborn rats on the 1^{st} to 3^{rd} day after the birth, under a microscope. Subsequently, such hippocampal portion were subjected to heat treatment with the digestive enzyme papain over ten minutes, and the resultant was then mechanically dispersed using a pipette, so as to obtain a hippocampal cell suspension that was rich in neurons. This suspension was diluted with a medium as necessary, and it was then inoculated onto a culture plate with a diameter of 35 mm, which had been coated with a cell adhesive substrate such as polylysine. The seeding density was set at approximately 20,000 to 40,000 cells/cm^2. These cells were adhered to the surface of the culture plate, and a primary culture was then carried out at a high density using an Eagle medium that contained fetal bovine serum and N2-supplement (an additive for neurons). Sixth or seventh days after initiation of the culture, 2 to 4 μg of DNA per 35-mm culture plate was introduced into the cells by the calcium phosphate method using a BDNF-mKO-FM14 expression gene vector at 37°C for 30 minutes. Approximately 12 hours to 2 days after introduction of this gene, a fluorescent protein expressing in the cells was detected under a fluorescence microscope, and it was then used in an experiment for tracing a change in color. 490DF20 (OMEGA) equipped with a 10% neutral density filter was used for green fluorescence signal excitation. 535DF35 (OMEGA) was used as a green fluorescence signal detection filter. On the other hand, 546DF10 (OMEGA) was used for orange fluorescence signal excitation. 595RDF60 (OMEGA) was used as an orange fluorescence signal detection filter. As a dichroic mirror, 505DRLPXR (OMEGA) was used. The

field stop was controlled, and only the orange fluorescence in the soma (cell body) of rat hippocampus neurons was discolored with the strong green light of 550DF30 (OMEGA). The ratio between the orange fluorescence signal and the green fluorescence signal was calculated, and the shift of BDNF-mKO-FM14 from the soma to the neurite was observed (Figures 33 and 34). The white arrow in Figure 34 indicates a state wherein BDNF-mKO-FM14 moves from the soma towards the tip of the neurite.

Example 7: Caspase-3 activity measurement probe using monomer fluorescent protein mKO and dimer (multimer) fluorescent protein MiCy

At least one type of protein used to perform intramolecular FRET should be a monomer. (A) the combination of the monomer (white) with the dimer (black) (Figure 35A). The combination of the dimer (polymer) fluorescent protein MiCy with the monomer fluorescent protein mKO corresponds to such a pattern. For example, it is considered that the combination of the dimer (white) with the dimer (black) causes a range such as a polymer (Figure 35B). In the case of the monomer fluorescent protein mKO and the dimer fluorescent protein MiCy, since the fluorescence spectrum of MiCy and the absorption spectrum of mKO partially overlap, it is possible to measure FRET (fluorescence resonance energy transfer method) using both proteins (Figure 36). Thus, MiCy is ligated to mKO, using a linker that contained DEVD (Asp-Glu-Val-Asp) as a Caspase-3 recognition sequence (the amino acid sequence thereof is shown in SEQ ID NO: 35, and the nucleotide sequence thereof is shown in SEQ ID NO: 36). Thereafter, the cleavage of the linker sequence due to activation of Caspase-3 was measured by FRET.

(1) *In vitro* measurement of Caspase-3 activity

MiCy, the linker, and mKO were ligated to one another in this order, and the obtained ligate was then subcloned into the BamH1-EcoR1 site of the *Escherichia coli*

expression vector $pRSET_B$, so that it was allowed to express in *Escherichia coli* JM109 (DE3). The used linker had the following sequence: GGSGGDEVDGTGGS (Gly-Gly-Ser-Gly-Gly-Asp-Glu-Val-Asp-Gly-Thr-Gly-Gly-Ser). This construct was referred to as MiCy-DEVD-mKO. The expressed recombinant fusion protein was purified with Ni-NTA agarose. The purified recombinant fusion protein was subjected to gel filtration using a sephadex G-25 column, and the buffer was substituted with a 150 mM KCl and 50 mM HEPES-KOH (pH 7.4) solution. For activity measurement, recombinant Active-Caspase-3 (MBL: BV-1083-9) was used. Each recombinant fusion protein was poured into a solution that contained 20 mM HEPES-KOH (pH7.4), 100 mM NaCl, 0.1% CHAPS, and 10% sucrose, resulting in a concentration of 1 mg/ml. Thereafter, 1 unit of the recombinant Active-Caspase-3 was added thereto, followed by reaction at 30°C for 3 hours. The fluorescence spectrum of the reaction solution was excited at 440 nm and measured before and after the reaction. For such measurement, a fluorospectrophotometer F-2500 (HITACHI) was used. As a result, it was found that FRET took place and the fluorescence peak (559 nm) of mKO appeared before addition of Caspase-3, but that after the addition thereof, FRET disappeared due to the cleavage of the linker and the fluorescence peak (559 nm) of mKO thereby disappeared, so that only the fluorescence peak (495 nm) of MiCy remained (Figure 37).

(2) *In vivo* measurement of Caspase-3 activity

MiCy-DEVD-mKO was subcloned into the BamH1-EcoR1 site of the animal cell expression vector pCS2+. The thus prepared vector was introduced into HeLa-S3 cells, using Polyfect (QIAGEN). Twenty-four hours after introduction of the gene, the culture solution was substituted with an HBSS (Hanks' Balanced Salt Solution) that contained 500 ng/ml anti-Fas antibody (CH-11: MBL) and 10 μg/ml cycloheximide, so as to induce apoptosis, followed by the imaging of Caspase-3 activity measurement.

IX-70 (OLYMPUS) was used as a microscope. 440AF21 (OMEGA) was used

as an excitation filter, and 455DRLP (OMEGA) was used as a dichroic mirror. A fluorescence signal was detected through the 480ALP (OMEGA) filter, using a color 3CCD camera ASHURA (Hamamatsu Photonics K. K.). The fluorescence signal of MiCy was detected in a green channel, and the fluorescence signal of mKO was detected in a red channel. As a result, as apoptosis proceeded in the HeLa cells, Caspase-3 was activated, and a linker of the translated product of the introduced gene was cleaved. Thereby, FRET disappeared, and the phenomenon whereby the signal in the red channel decreased and the signal in the green channel increased was observed. The ratio between red and green decreased, as Caspase-3 was activated. In addition, alteration of the shape due to the apoptosis of the HeLa cells was also observed (Figure 38).

Example 8: Isolation of novel chromoprotein gene from stony coral, preparation of novel fluorescent protein, and analysis of properties thereof

(1) Extraction of total RNA

A chromoprotein gene was isolated from coral. Montipora sp. was used as a material. A frozen Montipora sp. was crushed in a mortar, and 7.5 ml of "TRIzol" (GIBCO BRL) was then added to 1 g (wet weight) of the crushed Montipora sp. Thereafter, the obtained mixture was homogenized and then centrifuged at 1,500 x g for 10 minutes. Thereafter, 1.5 ml of chloroform was added to the obtained supernatant, and the mixture was then stirred for 15 seconds. Thereafter, the mixture was left at rest for 3 minutes. The resultant was then centrifuged at 7,500 x g for 15 minutes. Thereafter, 3.75 ml of isopropanol was added to the obtained supernatant, and the mixture was then stirred for 15 seconds, followed by leaving the mixture at rest for 10 minutes. Thereafter, the resultant was centrifuged at 17,000 x g for 10 minutes. The obtained supernatant was discarded, and 6 ml of 70% ethanol was added to the residue, followed by centrifugation at 17,000 x g for 10 minutes. The obtained supernatant was discarded, and the precipitate was then dissolved in 200 μl of DEPC water. Total RNA

dissolved in the DEPC water was 100 times diluted, and the values of O.D.260 and O.D.280 were then measured, so as to determine RNA concentration. As a result, 53 μg of total RNA was obtained.

(2) Synthesis of first strand cDNA

cDNA (33 μl) was synthesized from 4 μg of the total RNA, using a kit for synthesizing first strand cDNA "Ready To Go" (Amersham Pharmacia).

(3) Degenerated PCR

3 μl of the synthesized first strand cDNA (33 μl) was used as a template to carry out PCR. Primers were produced by making comparison among the amino acid sequences of known fluorescent proteins, extracting similar portions, and converting them to nucleotide sequences.

Primers used:

5'-GAAGGRTGYGTCAAYGGRCAY-3' (primer 1) (SEQ ID NO: 74)

5'-ACVGGDCCATYDGVAAGAAARTT-3' (primer 2) (SEQ ID NO: 75)

I represents inosine; R represents A or G; Y represents C or T; V represents A, C, or G; D represents A, G, or T; S represents C or G; H represents A, T, or C

Composition of PCR reaction solution:

Template (first strand cDNA)	3 μl
X10 taq buffer	5 μl
2.5 mM dNTPs	4 μl
100 μM primer 1	1 μl
100 μM primer 2	1 μl
Milli-Q	35 μl
taq polymerase (5 U/μl)	1 μl

PCR reaction conditions:

94°C x 1 min (PAD)

94°C x 30 sec (denaturation)

52°C x 30 sec (annealing of primers to template)

72°C x 1 min (primer elongation)

A cycle consisting of the aforementioned 3 steps was repeated 35 times.

72°C x 7 min (final elongation)

4°C (maintenance)

Using 1 μl of the amplified product obtained in the first PCR reaction as a template, PCR was carried out again under the same above conditions. A 350-bp fragment was cut out via agarose gel electrophoresis, and it was then purified.

(4) Subcloning and sequencing

The purified DNA fragment was ligated to a pT7-blue vector (Novagen). Escherichia coli (TG1) was transformed therewith, and it was then subjected to blue white selection. Plasmid DNA was purified from white colonies of Escherichia coli, and the nucleotide sequence of the inserted DNA fragment was determined using a DNA sequencer. Thereafter, the obtained nucleotide sequence was compared with the nucleotide sequences of other fluorescent protein genes, so as to determine whether the nucleotide sequence of the DNA fragment was derived from a fluorescent protein. With regard to those that were determined to be a part of the fluorescent protein genes, the full-length genes were cloned by the 5′-RACE method and the 3′-RACE method.

(5) 5′-RACE method

In order to determine the nucleotide sequence on the 5′-side of the DNA fragment obtained by the degenerated PCR, the 5′-RACE method was applied using 5′-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (GIBCO BRL).

5 μg of the total RNA prepared in (1) above was used as a template.

The following primers were used in the first amplification of dC-tailed cDNA:

5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3' (primer 3) (SEQ ID NO: 76); and

5'-CTCAGGGAATGACTGCTTTACAT-3' (primer 4) (SEQ ID NO: 77)

Herein, I represents inosine.

The following primers were used in the second amplification:

5'-GGCCACGCGTCGACTAGTAC-3' (primer5) (SEQ ID NO:78)

5'- GTCTTCAGGGTACTTGGTGA -3' (primer6) (SEQ ID NO:79)

PCR reaction conditions were applied in accordance with protocols attached to the kit.

The amplified 350-bp band was cut out of the gel via agarose gel electrophoresis and then purified. The purified DNA fragment was ligated to a pT7-blue vector (Novagen). Escherichia coli (TG1) was transformed therewith, and it was then subjected to blue white selection. Plasmid DNA was purified from white colonies of Escherichia coli, and the nucleotide sequence of the inserted DNA fragment was determined using a DNA sequencer.

(6) 3'-RACE method

The nucleotide sequence on the 3'-side of the DNA fragment obtained by the degenerated PCR was obtained by PCR using a primer prepared based on the information obtained by determination of the nucleotide sequence in (4) above and an oligo dT primer. 3 μl of the first strand cDNA prepared in (2) above was used as a template. The prepared primer was 5'- ATGTAAAGCAGTCATTCCCTGAG -3' (primer7) (SEQ ID NO: 80).

Composition of PCR reaction solution:

Template (first strand cDNA) 3 μl

X10 taq buffer	5 μl
2.5 mM dNTPs	4 μl
20 μM primer 7	1 μl
10 μM oligo dT primer	1 μl
Milli-Q	35 μl
Taq polymerase (5 U/μl)	1 μl

PCR reaction conditions:

94°C x 1 minute (PAD)

94°C x 30 seconds (denaturation)

52°C x 30 seconds (annealing of primers to template)

72°C x 1 minute (primer elongation)

A cycle consisting of the aforementioned 3 steps was repeated 30 times.

72°C x 7 minutes (final elongation)

4°C (maintenance)

The amplified band with a length of approximately 650 bp was cut out of the gel via agarose gel electrophoresis and then purified. The purified DNA fragment was ligated to a pT7-blue vector (Novagen). Escherichia coli (TG1) was transformed therewith, and it was then subjected to blue white selection. Plasmid DNA was purified from white colonies of Escherichia coli, and the nucleotide sequence of the inserted DNA fragment was determined using a DNA sequencer.

(7) Expression of protein in Escherichia coli

Based on the obtained full-length nucleotide sequence, a primer was produced with a portion corresponding to the N-terminus of the protein. An oligo dT primer was used as a primer corresponding to the C-terminal side thereof. Thereafter, using such primers, PCR was carried out employing the first strand cDNA prepared in (2) above as a template. The obtained full-length amino acid sequence and nucleotide sequence are

shown in SEQ ID NOS: 37 and 38 of the sequence listing. This protein having the amino acid sequence shown in SEQ ID NO: 37 was named COCP.

Primer used:

5'-CCCGGATCCGACCATGGCTACCTTGGTTAAAGA-3' (primer8) (SEQ ID NO: 81)

Composition of PCR reaction solution:

Template (first strand cDNA)	3 μl
X10 pyrobest buffer	5 μl
2.5 mM dNTPs	4 μl
100 μM primer 8	1 μl
100 μM oligo dT primer	1 μl
Milli-Q	35 μl
Pyrobest polymerase (5 U/μl)	1 μl

PCR reaction conditions:

94°C x 1 minute (PAD)

94°C x 30 seconds (denaturation)

52°C x 30 seconds (annealing of primers to template)

72°C x 1 minute (primer elongation)

A cycle consisting of the aforementioned 3 steps was repeated 30 times.

72°C x 7 minutes (final elongation)

4°C (maintenance)

The amplified band with a length of approximately 800 bp was cut out of the gel via agarose gel electrophoresis and then purified. The purified DNA fragment was subcloned into the BamHI-EcoRI site of a pRSET vector (Invitrogen), and it was then allowed to express in Escherichia coli (JM109-DE3). The expressed protein was constructed such that His-tag was attached to the N-terminus thereof, and thus it was

purified with Ni-Agarose gel (QIAGEN). Purification was carried out in accordance with the attached protocols. Subsequently, the properties of the purified protein were analyzed.

(8) Analysis of light-absorbing properties

Using a solution comprising 20 μM chromoprotein and 50 mM HEPES (pH 7.9), the absorption spectrum was measured. Thereafter, the molar absorption coefficient of the protein was calculated from the value of the peak of the spectrum. In the chromoprotein (COCP) derived from Montipora sp., the absorption peak was observed at 576 nm (Table 2, Figure 39. Also, it was stable at pH4 to 10.

Properties of chromoprotein (COCP) isolated from Montipora sp.

Table 1

	Maximum absorption	Maximum fluorescence	Molar absorption coefficient	Quantum yield	pH sensitivity	Number of amino acids
COCP	576 nm	-	64000 (576 nm)	-	Absent	221 a.a.
keima 616	440 nm	616 nm	28000 (440 nm)	0.24	Present	222 a.a.

(9) Modification from chromoprotein to fluorescent protein

COCP is not a fluorescent protein. However, valine was inserted into the portion between methionine at position 1 of COCP and serine at position 2 thereof, histidine at position 94 was substituted with asparagine, asparagine at position 142 was substituted with serine, asparagine at position 157 was substituted with aspartic acid, lysine at position 202 was substituted with arginine, and phenylalanine at position 206 was substituted with serine, so as to acquire fluorescence properties. This modified fluorescent protein was named as COCP-FL (the amino acid sequence thereof is shown in SEQ ID NO: 39, and the nucleotide sequence thereof is shown in SEQ ID NO: 40).

COCP-FL has an excitation peak at 560 nm. By this excitation, the fluorescence spectrum has a peak at 600 nm.

(10) Production of red fluorescent protein with large stokes shift

In COCP-FL, serine at position 62 was substituted with phenylalanine, isoleucine at position 93 was substituted with threonine, valine at position 124 was substituted with threonine, phenylalanine at position 159 was substituted with tyrosine, valine at position 192 was substituted with isoleucine, and serine at position 214 was substituted with alanine, so as to acquire a protein having different fluorescence from that of COCP-FL. This modified fluorescent protein was named as keima 616 (the amino acid sequence thereof is shown in SEQ ID NO: 41, and the nucleotide sequence thereof is shown in SEQ ID NO: 42). keima 616 has an excitation peak at 440 nm. By this excitation, the fluorescence spectrum has a peak at 616 nm (Figure 41, Table 2). Its stokes shift is 176 nm, which is an extremely large value. When compared with the conventional fluorescent protein, this protein is able to have a large excitation wavelength region and a large fluorescence wavelength region, and thus fluorescence can be efficiently measured. In addition, it is also possible to simultaneously measure multiple colors of fluorescence. Using fluorochromes having identical excitation wavelengths, photometry can be conducted with two wavelengths by excitation with a single wavelength such as a laser. In the case of the conventional fluorescent proteins, since proteins having the same excitation spectrum have not existed, such photometry has not been conducted. Using these proteins, a problem regarding deviation in measurement due to difference in excitation can be solved.

(11) Production of orange fluorescent protein having large stokes shift

In keima 616, phenylalanine at position 62 was substituted with methionine, and glutamine at position 63 was substituted with cysteine, so as to obtain a fluorescent

protein. This modified fluorescent protein was named as keima 570 (the amino acid sequence thereof is shown in SEQ ID NO: 43, and the nucleotide sequence thereof is shown in SEQ ID NO: 44). As with keima 616, keima 570 also has an excitation peak at 440 nm. By this excitation, the fluorescence spectrum has a peak at 570 nm (Figure 42). Its stokes shift is 130 nm, which is a large value. When compared with the conventional fluorescent protein, this protein is able to have a large excitation wavelength region and a large fluorescence wavelength region, and thus fluorescence can be efficiently measured. In addition, it is also possible to simultaneously measure multiple colors of fluorescence. Using fluorochromes having identical excitation wavelengths, photometry can be conducted with two wavelengths by excitation with a single wavelength such as a laser. In the case of the conventional fluorescent proteins, since proteins having the same excitation spectrum have not existed, such photometry has not been conducted. Using these proteins, a problem regarding deviation in measurement due to difference in excitation can be solved.

(12) Measurement of pH sensitivity

The absorption spectra of the proteins (keima 616 and keima 570) were measured in the following 50 mM buffer solutions (Figures 43 and 44):

The pH of each buffer solution is as follows:

pH 4, 5, and 5.5: acetate buffer

pH 6: phosphate buffer

pH 6.6: MOPS buffer

pH 7, 7.5, and 8: HEPES buffer

pH 9 and 10: glycine buffer

The peak value was stable between pH 7.5 and 10 (Figures 43 and 44).

Example 9

(1) Production of monomer red fluorescent protein having large stokes shift

In keima 616, leucine at position 61 was substituted with glutamine, threonine at position 93 was substituted with serine, threonine at position 124 was substituted with glutamic acid, tyrosine at position 189 was substituted with arginine, and tyrosine at position 191 was substituted with glutamic acid, so as to obtain a keima 616 protein as a monomer. This protein was considered to be a monomer keima 616 protein because its molecular weight, 30.1 kDa, obtained as a result of the ultracentrifugal molecular weight measurement, is almost the same as 29 kDa predicted from the amino acid sequence. This modified fluorescent protein was named as cmkeima 620 (Figure 45) (the amino acid sequence thereof is shown in SEQ ID NO: 45, and the nucleotide sequence thereof is shown in SEQ ID NO: 46). This protein has an excitation peak at 440 nm. By this excitation, the fluorescence spectrum has a peak at 620 nm (Figure 46). Its stokes shift is 180 nm, which is an extremely large value. When compared with the conventional fluorescent protein, this protein is able to have a large excitation wavelength region and a large fluorescence wavelength region, and thus fluorescence can be efficiently measured. In addition, it is also possible to simultaneously measure multiple types of fluorescence. Using fluorochromes having identical excitation wavelengths, photometry can be conducted with two wavelengths by excitation with a single wavelength such as a laser. In the case of the conventional fluorescent proteins, since proteins having the same excitation spectrum have not existed, such photometry has not been conducted. Using these proteins, a problem regarding deviation in measurement due to difference in excitation can be solved. Moreover, the entire molecular weight is suppressed, and there is no interaction due to multimer formation between fluorescent proteins. Accordingly, a change in the properties of labeled molecules can be reduced to the minimum.

(2) Modification of monomer red fluorescent protein having large stokes shift

Phenylalanine at position 62 of cmkeima 616 was substituted with leucine, so as to obtain a modified cmkeima 620 protein having an increased folding efficiency. This modified fluorescent protein was named as mkeima 620 (the amino acid sequence thereof is shown in SEQ ID NO: 47, and the nucleotide sequence thereof is shown in SEQ ID NO: 48). This protein has an excitation peak at 440 nm. By this excitation, the fluorescence spectrum has a peak at 620 nm. Its stokes shift is 180 nm, which is an extremely large value. Since this protein has fluorescence intensity that is relatively higher than that of cmkeima 620 (Figures 46 and 47), although this is a monomer, it is sufficiently usable, as with keima 616.

Example 10: Development of measurement system for one wavelength excitation two wavelengths photometry fluorescence cross-correlation spectroscopy, using protein with large stokes shift

Fluorescence cross correlation spectroscopy (FCCS) using fluorescent molecules is a method for measuring an intermolecular interaction. In this method, two fluorescent molecules are used as probes, so as to monitor an intermolecular interaction.

A great factor for deteriorating the sensitivity of cross-correlation in the currently used two wavelengths excitation FCCS measurement using two different fluorescent molecules is a deviation in the overlapped portions of two wavelengths measurement regions due to chromatic aberration. One wavelength excitation FCCS involving the combined use of fluorescent proteins capable of exciting with one wavelength and also separating fluorescence, such as keima 616 and ECFP, is able to avoid such a problem. Accordingly, an increase in the sensitivity of FCCS measurement is anticipated (Figure 48). Moreover, since such one wavelength excitation FCCS is able to avoid fluorescence resonance energy transfer (FRET), the measurement by FCCS is facilitated, and thus this method is suitable for detection of an interaction between proteins by FCCS. Accordingly, it is considered that, using keima

616 which is a fluorescent protein having a large stokes shift, detection of interaction between proteins can be easily and strongly carried out by FCCS.

(1) Detection of Caspase-3 activity

(a) Devices used for fluorescence cross-correlation measurement

TCS SP2 SOBS (Leica) and the FCCS system were used for fluorescence cross-correlation measurement. For EGFP-(spacer) DEVD-mRFP1, 458-nm Argon ion Laser and 594-nm HeNe Laser were used, and two wavelengths excitation was carried out. In addition, as the combination of ECFP with the keima 616 protein, 458-nm Argon Laser was used. Further, as light receiving band-pass filters, the following filters were used: EGFP: 500-550; mRFP1: 607-683; ECFP: 470-500; and keima 616: 535-585.

(b) Analysis of fluorescence cross-correlation measurement

The amino acid sequence DEVD which is cleaved with Caspase-3 was introduced into the portion between EGFP and mRFP and also into the portion between keima 616 and ECFP (Figure 49). The recombinant EGFP-DEVD-mRFP1 (x 2) (the amino acid sequence is shown in SEQ ID NO: 49, and the nucleotide sequence thereof is shown in SEQ ID NO: 50), ECFP-(spacer) DEVD-keima 616 (the amino acid sequence is shown in SEQ ID NO: 51, and the nucleotide sequence thereof is shown in SEQ ID NO: 52), and keima 616-(spacer) DEVD-ECFP (the amino acid sequence is shown in SEQ ID NO: 53, and the nucleotide sequence thereof is shown in SEQ ID NO: 54), were produced. Since the expressed proteins were constructed such that His-tag was attached to the N-terminus thereof, they were purified with Ni-agarose gel (QIAGEN). Purification was carried out in accordance with the protocols included therewith. Subsequently, these proteins were used to analyze cross-correlation.

For quantitative evaluation of cross-correlation, a value obtained by dividing the amplitude ($G_{cross}(0)$) of a cross-correlation function known as a relative amplitude by the

amplitude ($G_{lower}(0)$) of an autocorrelation function. In the case of EGFP-DEVD-mRFP1 (x 2), the value of $G_{cross}(0)$ /$G_{lower}(0)$ was approximately 0.4 (Figure 51). A decrease in $G_{cross}(0)$ was observed as a result of addition of Caspase-3 (Figure 50).

In the case of the combination of ECFP with keima 616, the value of $G_{cross}(0)/G_{lower}(0)$ was 0.4 (Figure 51). A rapid decrease in $G_{cross}(0)$ was observed as a result of addition of Caspase-3. A decrease in $G_{cross}(0)$ indicates that fluorescence correlation disappeared as a result of addition of Caspase-3. In the case of the combined use of ECFP with keima 616, such correlation disappeared in a shorter time than in the case of EGFP-DEVD-mRFP. From these results, it became clear that the combined use of ECFP with keima 616 exhibits the interaction between proteins more easily and rapidly by the fluorescence cross-correlation method.

(c) Analysis of interaction between proteins by SDS-PAGE

When keima 616-DEVD-ECFP was reacted with Caspase-3, the bands with the sizes of keima 616 and ECFP could be confirmed. The presence of such proteins means that DEVD was cleaved with Caspase-3 (Figure 52). In the case of Native-PAGE as well, two bands were confirmed after the reaction. The two bands were identified to be keima 616 and ECFP, and thus it was found that the activity of Caspase-3 could be detected also by fluorescence detection (Figure 52).

(2) Interaction with calmodulin

(a) Synthesis and expression of protein

ECFP was ligated to calmodulin, and keima 616 was ligated to M13 (Figure 53). The amino acid sequence of ECFP-calmodulin is shown in SEQ ID NO: 55, and the nucleotide sequence thereof is shown in SEQ ID NO: 56. In addition, the amino acid sequence of M13-keima 616 is shown in SEQ ID NO: 57, and the nucleotide sequence

thereof is shown in SEQ ID NO: 58. Such fusion proteins were allowed to express in the *Escherichia coli* strain (JM109-DE3). The expressied proteins were constructed such that His-tag was attached to the N-terminus. Thus, they were purified with Ni-agarose gel (QIAGEN). Purification was carried out in accordance with the protocols included therewith. Subsequently, these proteins were used to analyze cross-correlation.

(b) Devices for fluorescence cross-correlation measurement

ConfoCor2 (Carl Zeiss) and LSM 510, version 3.2, were used for fluorescence cross-correlation measurement. 458-nm Argon ion Laser was used. The following light receiving band-pass filters were used: EGFP: 475-525; and keima 616: LP610.

(c) Analysis of fluorescence cross-correlation measurement

For quantitative evaluation of cross-correlation, a value obtained by dividing the amplitude ($G_{cross}(0)$) of a cross-correlation function known as a relative amplitude by the amplitude ($G_{lower}(0)$) of an autocorrelation function. In the case of a sample obtained by chelating calcium ions with EGTA, the value of $G_{cross}(0)$ /$G_{lower}(0)$ was approximately 0.005 (Figure 54). However, an increase in $G_{cross}(0)$ was observed as a result of addition of calcium ions (Figure 55). These results show that calcium-dependent interaction between proteins was detected. From the results, it became clear that the interaction between proteins can be measured by the fluorescence cross-correlation method more rapidly and easily.

INDUSTRIAL APPLICABILITY

The present invention provides a novel fluorescent protein (mKO), which is able to exist in the form of a monomer. When the mitochondria of HeLa cells are labeled with a dimer fluorescent protein KO, the mitochondria are labeled in a granulated state,

and thus the original image of such mitochondria cannot be obtained. However, when the mitochondria are labeled with a monomer fluorescent protein mKO, the image of normal narrow filamentous mitochondria is obtained, and the dynamic movement thereof is also observed. Such effectiveness obtained by monomerization was confirmed by the labeling of mitochondrial molecules.

In addition, the fluorescent proteins (keima 616 and keima 570) of the present invention emit red and orange fluorescence, and the excitation peak thereof is 440 nm (blue). The conventional red fluorescent proteins (DsRed and HcRed) have a stokes shift (the difference between an excitation peak value and a fluorescence peak value) between 20 and 30 nm. In contrast, the red fluorescent protein of the present invention has a stokes shift of 176 nm, and the orange fluorescent protein of the present invention has a stokes shift of 130 nm. Thus, the fluorescent proteins of the present invention have extremely large values. Accordingly, the fluorescent protein of the present invention is characterized in that the maximum fluorescence can be obtained by the maximum excitation. Moreover, since the excitation peak is at 440 nm, in the simultaneous excitation staining with a cyan fluorescent protein (CFP) or a green fluorescent protein (GFP), it becomes possible to extremely effectively obtain the fluorescence of both proteins. Furthermore, the excitation peak of the conventional red fluorescent proteins is between 560 nm and 590 nm. In contrast, the fluorescent protein of the present invention has an excitation peak at 440 nm. Thus, by changing excitation light, it makes possible to stain the present fluorescent protein, simultaneously with the conventional red fluorescent protein.